CNS Drug Reviews
Vol. 13, No. 4, pp. 423–443

Cortagine: Behavioral and Autonomic Function of the Selective CRF Receptor Subtype 1 Agonist

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Keywords: Anxiety — Behavior — Cortagine — Corticotrophin — CRF1 agonist — CRH — Defense — Depression.

ABSTRACT

Corticotropin-releasing factor (CRF) is a neuropeptide and mediating component of neuroendocrine, autonomic, and behavioral processes associated with the stress response. The two receptor subtypes identified in the mammalian brain, CRF receptor subtype 1 (CRF1) and CRF2, are suggested to differentially modulate these processes. Manipulation of these receptors with selective CRF compounds and transgenic models has revealed, in most studies, a clear potentiation of the stress response through central activation of CRF1. However, pharmacological activation of CRF restricted to CRF1 has been limited by the availability of selective peptidic compounds. Recently, a highly selective CRF1 agonist, cortagine, has been developed. It was synthesized from chimeric intermediate sequences of ovine CRF, sauvagine, and human/rat CRF into a highly soluble peptide with strong affinity for CRF1 ($IC_{50} < 5$ nM) and a very low binding preference for CRF2 ($IC_{50} > 500$ nM). Affinity for the CRF binding protein ($IC_{50} > 1{,}000$nM) can be abolished by the addition of a glutamate residue on position 21 of the cortagine peptide sequence. Cortagine has recently been tested in a variety of preclinical models of behavior including the elevated-plus-maze (EPM), forced swim test (FST), homecage, and rat exposure test (RET). Preliminary characterization in the EPM and FST suggested that this compound elicits anxiogenic and antidepressant-like effects, respectively. Additional testing in the homecage and RET, which targets various elements of behavior, directs to a more potent anxiogenic profile of cortagine. In this review,

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Conflict of Interest: The authors have no conflict of interest.

we discuss the behavioral findings and the tests used to measure these effects. Finally, we also discuss preliminary findings of autonomic activation obtained by central injection of cortagine that support CRF1 involvement in the modulation of heart rate and heart rate variability.

INTRODUCTION

The effect of corticotropin-releasing factor (CRF)–related peptides on behavior have been studied extensively for more than 2 decades. Following its characterization in 1981 (Spiess et al. 1981; Vale et al. 1981), synthesis of agonists and antagonists has further developed a means for understanding the link between CRF activation and the behavioral output system of the stress response. Cortagine is the first CRF receptor subtype 1 (CRF1) peptide agonist developed by the Max Planck Institute for Experimental Medicine (Göttingen, Germany). This compound is being currently evaluated for its behavioral and autonomic role in the stress response. The scope of this article is to briefly describe the function of CRF in regulating the behavioral stress response and to review all available data on how the newly developed selective CRF1 agonist, cortagine, mediates these responses.

Mechanisms regulating the stress response include regions of the limbic system involved in the hypothalamic–pituitary–adrenal axis (HPA) and the autonomic nervous system (ANS) (Herman et al. 2005). The primary focus on the influence of CRF is evident by much of the early research with humans and animals that measured CRF activation of the HPA axis via circulating peripheral glucocorticoid levels (Antoni 1986; Whitnall 1993; see DeSouza and Nemeroff 1990). Yet, much of the research that uses manipulation of peripheral glucocorticoid levels including those that use CRF and related compounds, provide little evidence that the peripheral stress response influences anxiety and other related mood states (DeSouza and Nemeroff 1990). This suggests that while enhanced CRF release can produce dysregulation of the HPA axis (e.g., increase glucocorticoid circulation), which can be deleterious to physical health and function (Sapolsky 1992), it is manipulation of central activation of CRF that affects psychological states. This is supported by an abundance of research manipulating central CRF levels in a variety of animal models (Bale and Vale 2004; Lowry and Moore 2006; Valdez 2006), central nervous system concentration of CRF in human psychopathologies (Nemeroff et al. 1984; Banki et al. 1987; Nerozzi et al. 1988; Widerlov et al. 1988; Bremner et al. 1996; Heim et al. 1997; Arborelius et al. 1999; Bisette et al. 2003; Sautter et al. 2003), as well as current research evaluating CRF1 antagonists as an alternative treatment for depression (Zobel et al. 2000; Kunzel et al. 2003; Held et al. 2004).

CRF RECEPTOR DISTRIBUTION

Two CRF receptor subtypes, CRF1 and CRF receptor subtype 2 (CRF2), have been identified in the mammalian brain; these have a unique but fairly heterogeneous distribution that varies slightly when comparing different rodent and nonhuman primates (Chalmers et al. 1995; Sanchez et al. 1999; Van Pett et al. 2000; Lim et al. 2006). Homogeneity of receptor subtypes is found more commonly in subnuclei within specific structures such as

the septum, hypothalamus, amygdala, and raphe nuclei. CRF1 is found predominantly in the cortex, pituitary, central amygdala, thalamus, medial septum, brainstem, cerebellum, and sensory and motor regions (Chalmers et al. 1995; Van Pett et al. 2000; Kostich et al. 2004). CRF2 is found exclusively in subcortical regions including the medial amygdala, ventromedial nucleus of the hypothalamus, and lateral septum (LS). Moderate levels are found in other hypothalamic and amygdala nuclei, and the mesencephalic raphe (dorsal and median nuclei). Subregions within the hippocampal formation, including the entorhinal cortex, largely express both CRF receptor subtypes. CRF2 is further divided into splice variant forms, CRFR2α and CRFR2β. CRFR2α is found predominantly in the hypothalamus, LS, and olfactory bulb, and CRFR2β is distributed throughout the brain and peripheral tissue (Lovenberg et al. 1995).

The stresscopin-related peptides are included in the CRF family based on their high affinity for CRF receptors and consist of urocortin (UCN), urocortin II (UCN2), and urocortin III (UCN3) (Hsu and Hsueh 2001; Dautzenberg and Hauger 2002). Many of the agonists developed for pharmacological evaluation of CRF2 are of these ligands. UCN2 and UCN3 bind selectively to the CRF2 receptor, while UCN binds to both receptor subtypes. All bind to both splice variants of CRF2. UCN2 and UCN3 are predominantly distributed subcortically, with major sites of expression including the paraventricular, supraoptic, and arcuate nuclei of the hypothalamus, and the locus coeruleus of the rostral pons. Distribution in the motor nuclei of the brainstem (trigeminal, facial, hypoglossal), spinal ventral horn, has also been identified (Li et al. 2002; Cavalcante et al. 2006).

Abundant CRF expression throughout the primary regions of the brain linked to affective states and emotional processing, specifically the limbic system, (Gray 1990; Gray and McNaughton 2000), suggests that this neuropeptide may be a substrate responsible for exacerbating psychological stress. Current research is focused on elucidating the role of the two identified receptor subtypes in these functions. However, isolating receptor subtypes for evaluation is always limited by the availability of highly selective compounds.

BEHAVIORAL PHARMACOLOGY OF CRF1

Preclinical and Clinical Studies

CRF1 appears to be the primary receptor of CRF involved in activation of the HPA-axis response to stress (Contarino et al. 1999; Pellymounter et al. 2002; Lelas et al. 2004). CRF1 is well established as mediating a direct response to stress, including anxiety (Griebel et al. 2002; Gutman et al. 2003; Funk et al. 2007; Skelton et al. 2007), fear (Takahashi 2001), aggression (Farrokhi et al. 2004), and social behaviors (French et al. 2007). Sleep pattern disruptions in animals (Jones et al. 1998; Lancel et al. 2002) and humans (Held et al. 2002) have also been reported.

Until the first report on cortagine by Tezval et al. (2004), CRF1 agonists had not been available for pharmacological activation that was limited to this receptor. Most of the pharmacological manipulation of CRF1 has been through selective CRF1 antagonists and CRF agonist compounds such as ovine CRF (oCRF) and human/rat CRF (h/rCRF), which bind preferentially, but not selectively to CRF1. Many CRF1 antagonists are available in research; however, low solubility of the nonpeptidic compounds in aqueous solutions has made them problematic for central infusion. Most are infused orally or intraperitoneally (i.p.). The

abundance of research using these highly selective CRF1 antagonists in tests ranging from social interaction models (Gelhert et al. 2005; French et al. 2007), resident-intruder aggression model (Farrokhi et al. 2004), and forced swim test (Harro et al. 2001; Overstreet and Griebel 2004; Hodgson et al. 2007) have shown that they effectively and consistently reduce anxiety-like behavior. A CRF1 antagonist viable for human research, R121919 (2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylaminopyrazolo[1,5-a]pyrimidine), has also been developed and tested in patients with major depression. Initial clinical trials suggested that R121919 may reduce depressive symptoms with minimal side effects (Held et al. 2004; Kunzel et al. 2003; Zobel et al. 2000). However, there are unconfirmed reports that the drug was not properly metabolized, thereby posing a potential health risk, and clinical trials were halted. New orally active CRF compounds for clinical research are currently in the development phase.

This evidence in conjunction with transgenic studies of mice deficient for CRF1 (Smith et al. 1998; Cortarino et al. 1999; Gammie and Stevenson 2006) suggest that CRF1 activation would result in enhanced stress induced anxiety-like, autonomic, and neuroendocrine responding. However, pharmacological activation of CRF1 has been limited by the availability of selective peptidic compounds. The recent development of cortagine now allows direct manipulation of these systems and the potential effects on behavior and autonomic responding. We now review the development of cortagine and multiple preclinical models that have been used to evaluate its effects.

CORTAGINE: DEVELOPMENT

A group headed by Joachim Spiess at the Max Planck Institute for Experimental Medicine (Göttingen, Germany) made it possible to isolate and pharmacologically activate CRF1 by synthesizing the newly developed selective CRF1 agonist, cortagine.

Cortagine was derived from chimeric intermediate sequences of sauvagine (Svg), h/rCRF, and oCRF (Tezval et al. 2004; Todorovic et al. 2005). Peptides were divided into N-, central, and C-terminals and segregated at the N- and C-terminus to form multiple chimeric intermediates until the final sequence for cortagine was selected (Fig. 1). Svg was selected as the parent compound due to its strong affinity to both CRF1 and CRF2 and favorable solubility characteristics (Eckart et al. 2001). The central domain of h/rCRF (residues 14–30) was found to be responsible for reduced CRF2 binding, and was, therefore, selected for development of cortagine. Residue-40 of the C-terminal (Svg) was replaced with residue-41 of

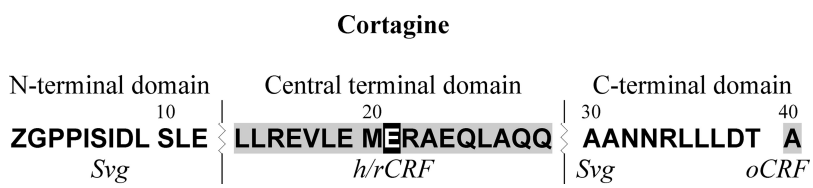


FIG. 1. Final amino acid sequence of cortagine. Cortagine consists of intermediate chimeric peptides derived from Svg, h/rCRF, and oCRF. A glutamate residue was introduced at position 21 to prevent binding to CRF binding protein (CRFBP). The sequence is separated at each domain of the N-, central-, and C-terminals. The parent amino acid sequence of each domain is as follows: N-terminal = Svg; central terminal = h/rCRF with replacement of Ala-21 with glutamate; C-terminal = Svg with replacement of residue 40 with Ala-41 of oCRF.

oCRF to produce a >5-fold increase in binding potential to CRF1. A final modification was made to reduce binding to CRFBP. This was achieved by replacing Ala-21 of the central terminal of h/rCRF with a glutamate residue. The final compound was based on its high affinity for CRF1 (average IC_{50} value = 2.6), and limited binding to CRF2 (average IC_{50} value = 540) and CRFBP (average IC_{50} value >1000).

Cortagine is highly soluble, with a maximum concentration in artificial cerebrospinal fluid (aCSF) of up to 1000 μM (see Tezval et al. 2004). Like other peptide solutions, frozen cortagine solution (-20°C) is stable, with a decline in stability under freeze–thaw cycling. Pharmacokinetic and toxicological data are not yet available. All solutions for centrally administered *in vivo* analysis have been obtained by diluting cortagine in a 5 mM acetic acid in (aCSF) solution (CH3COOH/1 × aCSF) maintained at 7.4 pH.

CORTAGINE AND BEHAVIOR

While published data on cortagine are scarce due to its recent synthesis, results with two widely used models of anxiety and depression, the elevated-plus-maze (EPM) and forced swim test (FST), respectively, have been published. Our laboratory has recently tested cortagine in a variety of animal models including the homecage test, and rat exposure test (RET). All tests were conducted with central infusion of this compound into the lateral ventricles (i.c.v.) and site specifically in the LS, dorsal hippocampus, and dorsal periaqueductal gray (dPAG) in various mouse strains. The following provides an overview of these models and the effects of cortagine in each test.

Elevated Plus Maze

Tezval et al. (2004) were the first to report effects of cortagine in the EPM and FST. The EPM is designed to measure anxiety-like behavior of rodents in an environment that allows exploratory behavior in two open arms of an apparatus and the option to retreat to a more secure environment in two closed arms. Exploratory behavior indicates a reduced anxiety state, whereas increased time spent in the closed arms suggests elevated anxiety levels.

Doses of 30, 100, and 300 ng (6.8–68 pmols) of cortagine administered i.c.v. decreased the amount of time spent in the open arms and the number of open arm entries. oCRF, a nonselective CRF agonist verified for exerting a robust anxiogenic response in a variety of animal models, was also tested in comparison to cortagine. Interestingly, oCRF produced the same anxiogenic effect at 100- and 300- ng doses, suggesting that cortagine is a more potent activator of these effects. To verify that cortagine exerts its effects through activation of CRF1 *in vivo*, Tezval et al. also infused these compounds into the intermediate zones of the LS. The LS is a region highly involved in anxiety modulation (Dielenberg et al. 2001; Sheehan et al. 2004) and expresses predominantly CRF2 (Van Pett et al. 2000). oCRF (100 ng) injected into this region produced the same anxiogenic profile as i.c.v. administration of both compounds in the EPM. Cortagine did not produce behavioral changes when infused intraseptally, confirming its *in vitro* selectivity for CRF1.

These results from cortagine in the EPM support the finding that CRF1 directly modulates anxiety-like responses in preclinical models of anxiety. CRF1-deficient mice and antagonists have consistently shown anxiolytic profiles in these models. Furthermore, the

comparison of effects with the nonselective agonist, oCRF, suggests that cortagine is a more potent substrate for inducing these anxiogenic effects.

Forced Swim Test

The effects of cortagine in the EPM are supported by the abundance of research showing that pharmacological or genetic blockade of CRF1 reduces anxiety-like behavior. However, Tezval et al. (2004) and Todorovic et al. (2005) found a paradoxical effect in the FST.

The FST is the most widely used, pharmacologically validated preclinical model of depression (Porsolt et al. 1977; Porsolt 1979; Petit-Demouliere et al. 2005). It involves placing a rodent in an inescapable water-filled container and measuring immobility, which indicates the level of coping and despair the animal expresses in a stressful environment. This model suggests that mobility, or the attempt to continue swimming as means of escape, indicates that an animal is actively coping within a threatening environment. A rodent that ceases or becomes increasingly immobile would indicate higher levels of despair or helplessness resulting in a disengagement of coping behavior. The FST involves a pretest swim trial 24 h prior to drug administration and testing.

Both the Tezval and Todorovic studies modified the model and looked at cortagine administration in the FST before the preswim trial and prior to the swim test. This was done for the purpose of verifying whether immobility differences on the test day were potentially influenced by drug-induced learning interference of preswim training rather than antidepressant-like effects. In both studies, cortagine, administered i.c.v. at 30–300 ng, decreased immobility time compared to controls in either condition. The nonselective agonist, oCRF, also attenuated immobility, but at higher doses compared to cortagine (100 and 300 ng). Interestingly, 300 ng cortagine, but not oCRF also produced a long-lasting effect on immobility that was observed 24 h following drug injection. When infusion of cortagine was limited to the dorsal hippocampus, immobility was reduced similar to i.c.v. infusion, albeit the effect was markedly smaller (Todorovic et al. 2005). This suggests that the dorsal hippocampus confirms and is likely to be involved in the paradoxical antidepressant-like effects of cortagine in the FST.

Based on the FST as an antidepressant drug screening model, these results suggest that cortagine produces antidepressant-like behavior. This is in contrast to FST studies that found antidepressant-like effects by central CRF1 antagonism. Hodgson et al. (2007) tested a single dose of the CRF1 antagonist CP-154,526 (*N*-butyl-*N*-ethyl-4,9-dimethyl-7-(2,4,6-trimethylphenyl)-3,5,7-triazabicyclo[4.3.0]nona-2,4,8,10-tetraen-2-amine) and reported a decrease in immobility time in the FST. By a 2-week long chronic administration SSR125543 [4-(chloro-4-methoxy-5-methylphenyl)-*N*-[(1*S*)-2-cyclopropylfluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazamine] also elevated mobility in the FST (Overstreet and Griebel 2004). Bale and Vale (2003) looked at the effect of the unselective CRF receptor antagonist antalarmin in CRF2 deficient mice, which characteristically show decreased immobility in the FST compared to their wild-type littermates, and also reported that the CRF1 antagonist decreased immobility time. While results from these studies would suggest that a CRF1 agonist should produce a suppression of swimming in the FST, cortagine produced the elevated mobility response similar to the CRF1 antagonists. This may indicate that cortagine differentially modulates anxiety and depressive-like behaviors producing antidepressant-like effects similar to the CRF1

antagonists. However, another viable explanation is that the FST may not be differentiating between locomotor and antidepressant-like effects of this CRF agonist, particularly since oCRF was reported to produce a similar reduction in immobility when infused immediately before testing.

Todorovic et al. (2005) provide a reasonable explanation as to why this is not the case: Locomotor effects depended on the model employed. Cortagine in the EPM decreased the distance traveled, and so reduced mobility. Based on this decrease of mobility in the EPM, it would not be sufficient to suggest that the effects of cortagine in the FST are simply locomotor effects. However, Blanchard and Blanchard (1990) and Blanchard et al. (2001) explain that interpretation of behaviors also depends on the model employed. They suggest that anxiety-like or defensive responses change depending upon the source and proximity of, and the options available in dealing with, a threat source. For example, they have shown that if an animal is being approached, it will flee if an escape route is available, or freeze if there is not. One instance will demonstrate an increase in mobility while the other produces the opposite effect; however, the interpretation that fear is elevated should be the same in both tests. A more potent stress source with limited options for responding such as the FST allows two options: increase the swim rate to attempt escaping or fleeing or become immobile. The EPM provides many more options and poses a more looming threat that provides the option of retreat to an area of relative safety rather than struggling to escape if there is a threat source. Thus, comparing the measure of mobility in both tests as an index for emotionality may not be appropriate.

While the FST may be a good screening model for antidepressant effects of drugs, it may be a weak indicator of emotionality with compounds that also produce locomotor effects or high levels of anxiety, such as those that may induce panic-like behaviors. Perhaps, in some cases, the increase in mobility may be indicating an increase in escape attempts due to elevated anxiety-like behavior instead of coping. As with all preclinical models of human behavior, this model does have shortcomings as a screening device for antidepressant compounds, albeit it remains the most widely used model for this purpose.

Homecage Test

Based on these previous published results with cortagine, our laboratory was interested in whether CRF1 activation produces aberrant effects on behavior compared to the distinctive anxiogenic profile of nonselective CRF activation and antidepressant-like effects of specific CRF1 antagonists. To verify the anxiogenic effects in the EPM and further explore the unexpected direction of cortagine in the FST, our laboratory used two additional paradigms that provide very distinct components of behavior in the mouse: the homecage test and the RET.

The homecage test permits evaluation of acutely administered compounds on a range of behaviors emitted by subjects without further disruption over a 3-h time span. This enables a fine analysis of behavioral changes over time, following a single injection, on nondefensive behaviors such as feeding, locomotion, and sleep patterns, as well as on potentially occurring anxiety-like or defensive behaviors. The latter, typically not observed in the homecage, provides a very sensitive baseline for detecting enhanced defensive behavior, such as increased freezing. Furthermore, analysis of the duration and pattern of behavioral effects over time can provide a range for optimal testing periods following a single central injection.

Our laboratory observed the effects of cortagine in the homecage during the inactive phase to observe potential disruptions in a wide range of behaviors including; sleep patterns, a characteristic attributed to CRF elevation (Jones et al. 1998; Lancel et al. 2002; Held et al. 2004; Borna Farrokhi et al. in press); activity, shown both to increase and decrease in familiar environments using nonselective CRF agonists (Buwalda et al. 1997; Jones et al. 1998; Valdez et al. 2002; Borna Farrokhi et al. in press), and defensive or anxiety-like behaviors including freezing, a characteristic observed with CRF elevation in the homecage (Borna Farrokhi et al. in press). In the evaluation of cortagine, animals were placed in the homecage immediately following a single i.c.v. infusion of cortagine (0, 100, 500 ng) and observed for 3 h. A time-sampling method was used to record behaviors at each 1-min mark after which 20-min time bins were created to form 9 time points along a continuum.

Cortagine administered i.c.v. in the homecage produced a robust dose-dependent disruption of sleep (Fig. 2A). As the number of sleep observations began to stabilize in controls approximately 40 min following drug infusion, the cortagine infused animals showed a clear suppression at the 60-min time bin that lasted until approximately 100 min into testing for the low dose (100 ng) and 140 min for the high-dose group (500 ng). In the absence of sleep, the high-dose group of cortagine exhibited predominantly a noncharacteristic behavior in the homecage: freezing (Fig. 2B). In a familiar environment and in the absence of a threat source, as is found in most animal models of emotionality, freezing is not a typical

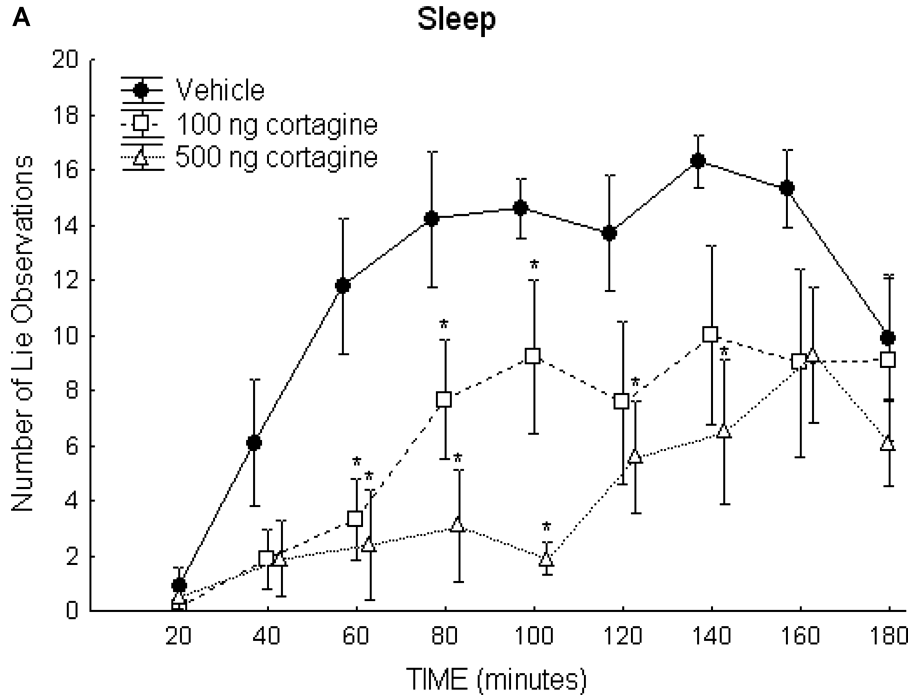
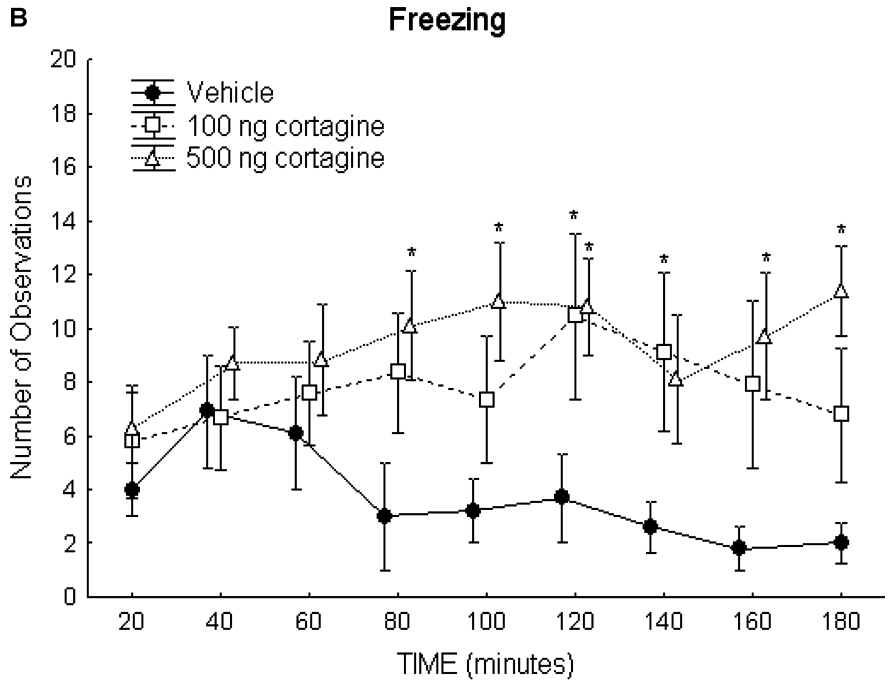


FIG. 2. Effects of cortagine by i.c.v. administration in the homecage test. Cortagine homecage test results were measured by the time-sampling method. Behaviors were observed every minute for 1 second and recorded. Nine time points were created by collapsing every 20 min into one time bin. Significant findings of behaviors measured in the homecage test included sleep (A), freezing (B), locomotor activity (C). Significant data are shown by comparing drug dose groups with controls where * indicates $P < 0.05$.



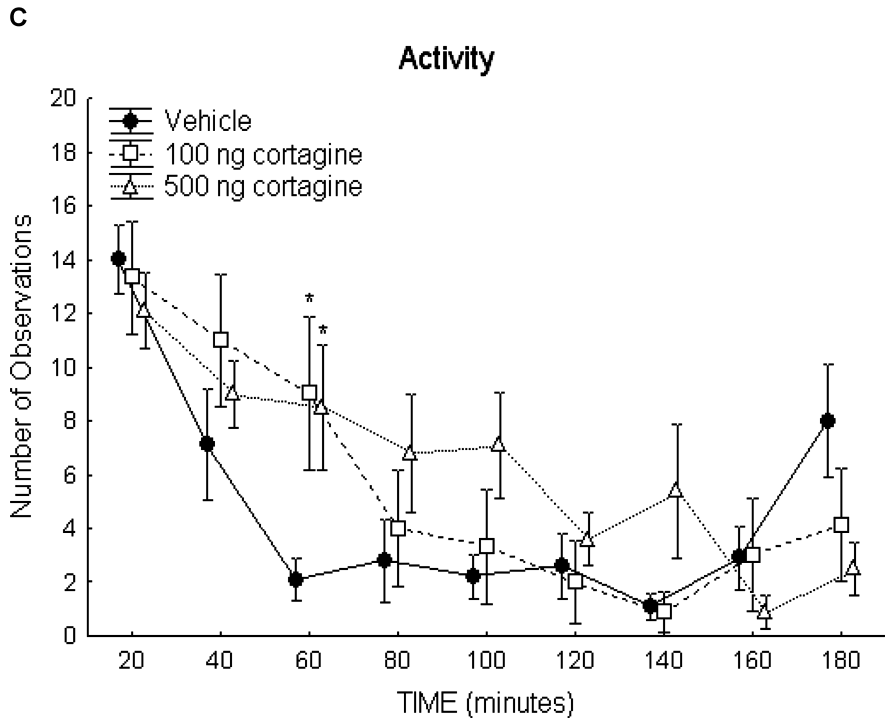


FIG. 2. Continued

behavior expected to emerge. However, in comparison to controls, high levels of freezing were reported with the cortagine infused animals (500 ng) in the homecage at approximately 80 min and continued until the third hour.

We also found that both high and low doses of cortagine produced a brief elevation in locomotor activity that was distinguishable from controls at the 60-min time bin (Fig. 2C). Locomotor activity was another facet explored, since locomotor effects vary and are reported to be conditional upon familiarity of the environment following pharmacological elevation of CRF. Locomotion is shown to be inhibited in novel environments and enhanced in familiar environments in rats (Menzaghi et al. 1994; Buwalda et al. 1997; Jones et al. 1998; Valdez et al. 2002). In contrast to previous reports on the effects of CRF agonists in a familiar environment, there may be some further discrepancies (i.e., potential species differences), since observation of oCRF effects in homecage of primates (Kalin et al. 1983) and in our studies with mice (Borna Farrokhi et al. in press) actually inhibited locomotor activity.

The sleep-pattern disruption of cortagine in the homecage was consistent with previous reports that CRF agonists and CRF1 antagonists alter sleep activity. However, the clear emergence of freezing behavior in the homecage suggests that CRF1 elevation with cortagine is sufficient to produce a robust anxiogenic effect, even under minimally threatening conditions. Furthermore, cortagine appears to produce a slight increase in locomotor activity, which is differentiated from its nonspecific counterpart, oCRF, which suppressed these effects in the identical paradigm.

Rat Exposure Test

Antipredator defense is sensitive to a variety of anxiolytic, anxiogenic, and antidepressant compounds (Blanchard et al. 1993a, 1993b, 1997; Griebel et al. 1995a, 1995b; Carvalho-Netto et al. 2007). Most traditional models of anxiety and depression have demonstrated their utility and pharmacological validity through consistent evaluation of behavior. However, many require learning (i.e., shock) or artificial stimuli that can disguise the source of fear or anxiety (i.e., footshock: response to pain or fear? EPM: fear of elevation or open space? FST: immobility as helplessness or adaptive learning? etc.). Investigating ethologically relevant defensive responses can help target innate processes of emotionality in mammals by creating a seminatural condition and eliciting responses pertinent to the natural habitat of the species.

The RET is a model that measures these defensive responses in a variety of inbred and outbred laboratory mice (Yang et al. 2004). It measures behaviors of mice toward a rat (potential predator) in a situation that permits a wide range of defenses, including avoidance, risk assessment, and freezing (Yang et al. 2004; Blanchard et al. 2005). This model provides a number of options for subject mice: They may stay on a surface area in proximity to the stimulus rat; escape to a tunnel attached to the surface area; flee to a chamber area farthest from the stimulus; freeze or exhibit a wide range of risk-assessment behaviors involving stretch postures toward a threat source to assess its danger potential. A detailed description of the model is presented in Blanchard et al. (2005).

We were interested in the effects of cortagine in this model to examine how pharmacological elevation of CRF1 modulates these innate defensive behaviors compared to nonspecific CRF activation. In CD-1 mice, a 500-ng dose of cortagine infused i.c.v. enhanced freezing

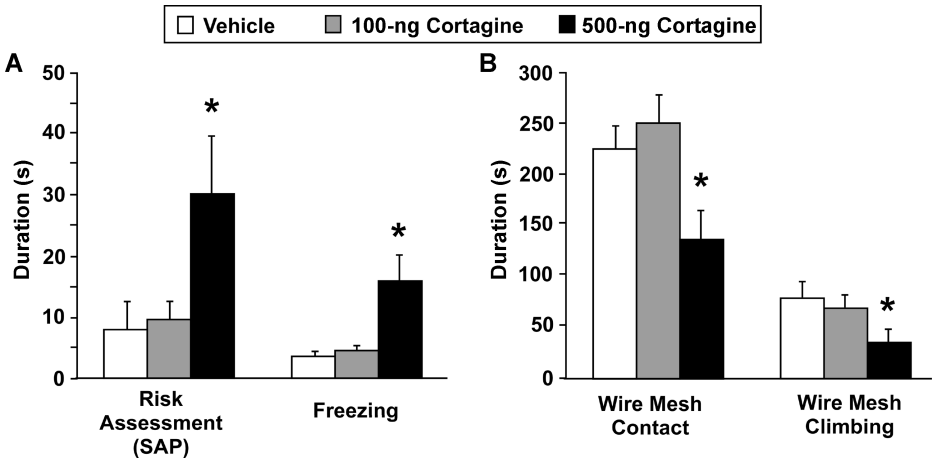


FIG. 3. Effects of cortagine by i.c.v. administration in the rat exposure test (RET). The following graphs represent data for the RET following i.c.v. infusion of 0, 100, and 500 ng of cortagine. Freezing was measured as the cessation of all movement. Risk assessment was a measure of stretch-attend posture where the mouse orients and elongates toward a potential threat stimulus (A). Avoidance measures are depicted by the amount of time spent in contact, or climbing of, the wire mesh separating the mouse from the stimulus rat (B). Significant data are shown by comparing drug-dose groups with controls where * indicates $P < 0.05$.

and risk assessment (Fig. 3A). Cortagine also increases avoidance indicated by a reduction in the amount of time mice spent in contact with the wire mesh separating the mouse from the stimulus (Fig. 3B). However, other avoidance measures determined by the spatial location of the mouse relative to the rat (surface versus chamber time) were not significant. Previous examination with the nonspecific CRF agonist, oCRF in the RET, both i.c.v. (Borna-Farrokhi et al. in press) and into the dPAG (Carvalho-Netto et al. 2007), produced a potent increase in defensive responses. In both tests, oCRF induced high levels of avoidance and by i.c.v. administration also produced a robust enhancement of freezing. Avoidance measures with cortagine were not as robust as those observed with oCRF. This suggests that CRF1 plays a significant role in anxiety elevation, but there appears to be a more potent, elevation with general CRF activation.

However, site-specific infusion of cortagine in the dPAG suggests that cortagine produces the same, if not a more potent, elevation in defensiveness. The midbrain periaqueductal gray (PAG) is a region with significant involvement in fear, anxiety (Behbehani 1995), and innate defensive behavior, particularly in antipredatory responses (Canteras and Goto 1999; Comoli et al. 2003). Both CRF1 and CRF2 neurons are expressed in the PAG (Van Pett et al. 2000), and CRF has been shown to have an excitatory effect on PAG neurons *in vitro* (Bowers et al. 2003).

Litvin et al. (2007) recently demonstrated that at 100 ng, cortagine produced the same robust spatial avoidance as oCRF in the RET at the same dose (Fig. 4). The intra-dPAG infusion of cortagine produced a more than two-fold increase in spatial preference of the chamber area farthest from the stimulus. Importantly, there were no differences in locomotion among groups to suggest that spatial preference durations were confounded by activity levels. In addition to an increase in avoidance, cortagine also significantly elevated defensive burying: a behavior attenuated by the CRF antagonists, D-phe CRF(12–41), (Basso et al. 1999) and positively related to anxiety levels (De Boer and Koolhaas 2003).

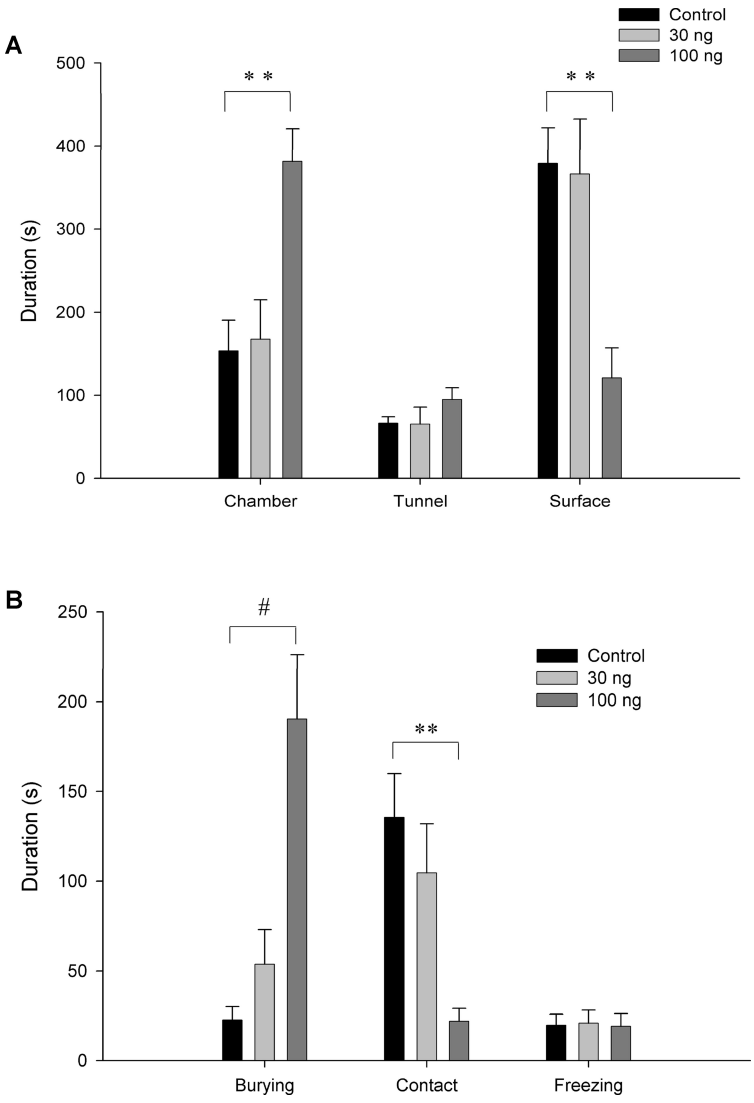


FIG. 4. Effects of cortagine by intradorsal periaqueductal gray (dPAG) administration in the rat exposure test (RET). The following graphs represent data for the RET following dPAG infusion of 0, 30, and 100 ng of cortagine. Avoidance (A) was determined by the amount of time mice spent in each location (chamber, tunnel, surface) of the apparatus relative to a stimulus rat, which is partitioned with a wire mesh on the surface area. Enhanced avoidance is determined by a decrease in surface and an increase in chamber duration. Graph (B) depicts the average amount of time mice spent burying, in contact with the wire mesh, and freezing. Significant data are shown by comparing drug-dose groups with controls where # indicate $P < 0.001$, ** indicates $P < 0.01$, and *indicates $P < 0.05$ (Litvin et al. 2004).

Results from i.c.v. and dPAG administration of cortagine in the RET suggest that activation of CRF limited to the type-1 receptor is sufficient to produce a potent anxiogenic response. These behaviors expressed through enhanced defensiveness toward a potential predator support the specific role of CRF1 in elevating these innate responses.

CORTAGINE AND AUTONOMIC FUNCTION

While much of the focus on CRF1 relates to its modulatory function on anxiety and depression, its role in regulating the ANS is equally important based on the potential health risks associated with alterations in CRF-induced functions including heart rate and blood pressure. The role of CRF in autonomic function has been extensively investigated. Elevated cardiac output and blood pressure is thought to be mediated by central CRF administration in rats, whereas peripheral administration results in vasodilation, lowered blood pressure, and, consequently, increasing heart rate (Overton and Fisher 1991; Richter and Mulvany 1995; Parkes et al. 2001). Recent studies using selective CRF receptor ligands and mutant mouse models point at a more complex regulation of autonomic function by the CRF system.

In contrast to the findings obtained in rats, Stiedl et al. (2005) suggested that central mediation of CRF1 functions can reduce baseline heart rate and attenuate tachycardic responses to an aversively conditioned auditory stimulus. Using a tone-shock dependent classical conditioning model, they were able to obtain baseline heart rate and fear-induced heart rate changes following conditioning. At the time these data were collected, selective CRF1 agonists were not available. Instead Stiedl et al. compared the CRF1 preferentially binding compounds, oCRF and human/rat CRF (h/rCRF), with the selective CRF2 agonist, UCN2. Nonselective and specific CRF2 antagonists were also evaluated to see whether these effects could be blocked. They found that oCRF and h/rCRF (210 and 170 ng, respectively), produced baseline bradycardia, attenuated fear-induced tachycardia, and increased heart rate variability, while UCN2 did not produce any changes in heart rate in either condition. Central injection of h/rCRF in mutant mice deficient for CRF2 resulted in similar changes of heart rate dynamics compared with their littermate wild-type controls. To further evaluate receptor modulation, pretreatment with the nonselective CRF antagonists, α-helical CRF, acidic astressin, or antisauvagine, a potent and selective CRF2 antagonist, were administered to determine if blunted tachycardic effects of the agonists following a conditioned stimulus could be blocked. Both nonselective antagonists returned elevated heart rate and variability to control levels, whereas antisauvagine failed to block these effects. Furthermore, mice lacking functional CRF2 display intact heart rate responses during novelty exposure and retention of conditioned auditory fear (Stiedl et al. 2003).

From these data they were able to eliminate centrally mediated CRF2 activation as involved in these changes. Instead, they concluded that vagal overactivation and enhanced sympathovagal antagonism mediated by central activation of CRF1 receptors were the mechanisms underlying the relative bradycardia, attenuated stress-induced increases in heart rate, with concomitant enhanced heart rate variability. Recently, Tovote et al. (unpublished data) repeated auditory fear conditioning paradigm in C57BL/6N mice using cortagine, and confirmed the results obtained earlier. By i.c.v. injections cortagine dose-dependently reduced baseline heart rate and attenuated conditioned tachycardia, while heart rate variability was strongly enhanced (Fig. 5).

Blood pressure has been reported to be a function of CRF2 mediation (Coste et al. 2000; Chen et al. 2003). Rivier et al. (2007) have recently tested arterial blood pressure effects of intravenous cortagine and stressin$_1$-A, another recently developed CRF1 agonist. Stressin$_1$-A binds with equal affinity to CRF1 and oCRF, and is reported to have a four-fold greater affinity for the CRF1 receptor over CRF2. Physiological analysis of stressin$_1$-A *in vitro* and *in vivo* has been conducted with rats, but behavioral testing has yet to be reported. As expected, this compound has shown to increase ACTH release, a function mediated by CRF1

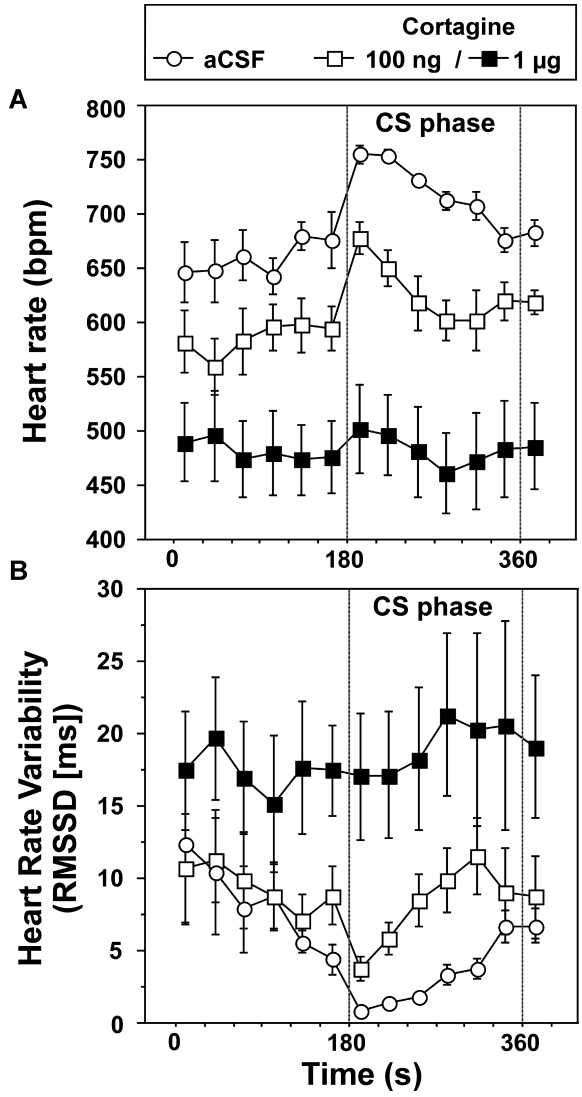


FIG. 5. Heart rate measures following i.c.v. administration of cortagine. The top graph (A) depicts heart rate (HR) in beats per min (bpm) during a 3-min baseline period and following presentation of a 3-min conditioned auditory fear stimulus during the CS phase. The bottom graph is a time domain measure of heart rate variability (B) calculated as the root mean square of successive RR interval differences (RMSSD). Cortagine dose-dependently decreased baseline HR and attenuated the conditioned HR increase. HR variability was markedly enhanced by cortagine.

(Smith et al. 1998; Timpl et al. 1998; Li et al. 2005) both *in vitro* and *in vivo*. The ACTH response *in vivo* (i.v.) was greater with oCRF at 10 min (5.0 μg/kg) following exposure, but stressin$_1$-A (5.0 μg/kg) continued to upregulate ACTH 3 h, later, while oCRF effects diminished at this time point. A 10 μg/kg dose (i.p.) of stressin1-A also increased, colonic transits with no effect on gastric emptying.

Neither cortagine (Tovote and Spiess, unbublished) nor stressin$_1$-A (3, 10, and 30 μg/kg) produced changes in arterial blood pressure when administered peripherally. These findings provide further evidence for CRF1 selectivity of both compounds, since CRF-mediated hypotension in the periphery is thought to be a function of CRF2.

SUMMARY AND FUTURE DIRECTION

Table 1 is a summary of the results obtained in behavioral and autonomic studies of cortagine. Cortagine is the first selective peptidic CRF1 agonist, and current behavioral evaluation suggests that it modulates behavior slightly differently than what is expected based on previous research with CRF1 antagonists and transgenic models. First, preliminary studies with the EPM, homecage, and RET show that pharmacological activation of CRF1 is sufficient to exert a potent anxiogenic response on classic anxiety-like, baseline, and ethologically relevant defensive behaviors similar to amalgamated CRF activation. Next, the paradoxical effects of cortagine in the FST point toward a potential for CRF1-mediated antidepressant effects.

The prospective of cortagine as a mixed agonist–antagonist or partial agonist accounting for these antidepressant-like effects cannot be excluded. However, this is unlikely because of its characterization as a potent cAMP releaser in the CRF1 transfected HEK cell system and in comparison with the potent CRF agonist, oCRF (Tezval et al. 2004). Unspecific affinity to and action through different, unknown receptors cannot be excluded, but that holds true for all ligands.

The concept of an antidepressant-like effect of a CRF1 agonist seems contrary to what is known about CRF function in depression and the antidepressant potential and efficacy of CRF1 antagonists currently in clinical trials. However, cortagine was tested under acute conditions, and it is conceivable that this CRF1 agonist may exert differential effects on depressive-like behaviors following single versus chronic administration, independent of its effects on anxiety-like behaviors. For example, certain compounds including selective serotonin-reuptake inhibitors (SSRIs) have been shown to produce nearly opposite effects depending on whether administration was acute or chronic (Griebel et al. 1995; Bagdy et al. 2001). Studies involving chronic administration of cortagine have not yet been conducted but may help to elucidate its role in modulating depressive-like behaviors.

Since CRF1 antagonists have demonstrated antidepressant effects repeatedly in preclinical (Bale and Vale 2003; Overstreet and Griebel 2004; Hodgson et al. 2007) and human studies (Zobel et al 2000; Kunzel et al. 2003; Held et al. 2004), another explanation for these elevated mobility effects is that the FST may be demonstrating weak discriminant validity for identifying behaviors beyond antidepressive-like behavior. In this case, one plausible theory for the increase in mobility with cortagine is that it may be identifying a robust anxiogenic or "panic-like" response that enhances rather than decreases locomotor activity in the FST. In a "panic-like" state, animals might attempt to escape or flee (Griebel et al. 1995a, 1996), which would also result in elevated mobility.

Further behavioral testing in additional preclinical models of depression such as the learned helplessness (Maier and Seligman 1976) or tail suspension (Steru et al. 1987) test will also help clarify the effects of cortagine on depressive-like behaviors.

Additional physiological characterization may include the central effects of cortagine on arterial blood pressure measures to compare with its failure to alter blood pressure following

TABLE 1. Summary of studies with cortagine.
The following is a summary table of all cortagine research available in publication or currently in preparation

Test (route)	Animal strain	Dose of cortagine	Result	Reference
EPM				Test
Lateral ventricles (i.c.v.)	C57BL/6J	30–300 ng (6.8–68 pmol)	Anxiogenic	Tezval et al. 2004; Todorovic et al. 2005
Lateral septum	C57 \ BL/6J	100 ng (21 pmol)	No effect	Tezval et al. 2004
FST				
(i.c.v.)	C57BL/6J, CRF_2 −/−,	30–300 ng 300 ng	Antidepressive	Tezval et al. 2004; Todorovic et al. 2005
Dorsal hippocampus	C57BL/6J	300 ng	Antidepressive	Todorovic et al. 2005
Homecage (i.c.v.)	CD-1	500 ng (210 pmol)	Anxiogenic, sleep distruption	Borna Farrokhi et al., unpublished data
RET				
(i.c.v.)	CD-1	500 ng	Anxiogenic, ↑ defense	Borna Farrokhi et al., unpublished data
Dorsal periaqueductal gray	Swiss Webster	100 ng	Anxiogenic, ↑ defense	Litvin et al. 2007
Autonomic Response				
Heart rate (i.c.v.)	C57BL/6J	100 ng, 1 μg	↓ Heart rate, ↑ variability	Tovote et al. unpublished data
Blood pressure (i.v., i.p.)	Sprague-Dawley, C57BL/6J	3–30 μg/kg,	No effect	Rivier et al. 2007. Tovote et al., unpublished data

peripheral administration. This can determine if CRF1 is involved in the mediation of arterial blood flow similar to CRF2 and to verify that the effects on autonomic function are centrally mediated.

Aside from the significant behavioral disruptions produced by exogenous CRF1 activation, the parasympathetic overactivation and enhanced sympathovagal antagonism produced by CRF1 agonists including cortagine, have been shown to produce autonomic dysregulation. Elevated arrhythmic risk can be a result of this enhanced sympathovagal antagonism, and has been implicated in sudden cardiac death. The study of selective CRF1 agonists provides critical information on the impact of this receptor in dysregulation of autonomic function and behavior, and has revealed a complex role of CRF1 in the orchestration of stress responses. While there are an abundance of selective CRF1 antagonists available, they are nonpeptidic in nature and fairly limited to peripheral administration. Although cortagine is not yet commercially available, its development now enables further selective manipulation of (CRF1) centrally to better understand the behavioral function of this receptor and its role in homeostatic function.

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